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DOI:
10.1039/c7mt00254h

Document Version
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Citation for published version (APA):
Schlottmann, F., Vera-Aviles, M., & Latunde-Dada, G. O. (2017). Duodenal cytochrome *b* (Cybrd1) ferric reductase functional studies in cells. *Metallomics*, *9*(10), 1389-1393. https://doi.org/10.1039/c7mt00254h

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Download date: 05. May. 2023

DUODENAL CYTOCHROME B (CYBRD1) FERRIC REDUCTASE: FUNCTIONAL STUDIES IN CELLS

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Abstract

Dietary non-heme ferric iron is reduced by the ferric reductase enzyme, duodenal cytochrome b (Dcytb), before absorption by the divalent metal transporter 1 (DMT1). A single nucleotide polymorphism (SNP rs10455 mutant) that is located in the last exon of Dcytb gene was reported in C282Y haemochrotomatosis HFE subjects. The present work therefore investigated the phenotype of this mutant Dcytb in Chinese hamster ovary (CHO) cells. These cultured cells were transfected with either wild type (WT) or the SNP vector plasmids of Dcytb. Ferric reductase assays were performed in Dcytb transgenic CHO cells using the ferrozine spectrophometric assay protocol. Furthermore, expression of the protein was confirmed by Western blot analysis. The Dcytb SNP rs10455 showed a gain-of-function capability since ferric reductase activity increased significantly ($P<0.01$) in the transgenic cells. Increased ferric reductase activity was found when CHO cells were pretreated with modulators of Dcytb protein expression. Although ferric reductase in endogenous CHO cells increased with deferoxamine or $CoCl_2$, iron loading with ferric ammonium citrate (FAC) had the opposite effect. Taken together, the study reveals a gain-of-function phenotype for Dcytb rs10455 mutation that could be a putative modifier of colorectal cancer risk, with attendant variability in penetrance among human HFE C282Y homozygotes.

Keywords: Dcytb, iron, ferric reductase, hypoxia, HFE, SNP

Introduction

Duodenal cytochrome b (Dcytb) was identified and characterized[1] as an iron-regulated ferric reductase localized primarily in the duodenal brush-border membrane[1]. The enzyme consists of 286 amino acids with six trans-membrane domains and is expressed in the brush border enterocytes. Dcytb shares 45-50% sequence homology with cytochrome b_{561}, a member of the family of oxidoreductases that is involved in ascorbate-mediated trans-membrane electron transport. Its expression has also been reported in the lungs[1;2], human erythrocytes[3] and in the brain[4]. Dcytb functions as a reductase in the conversion of ferric to ferrous ion for apical membrane import[1] by the divalent metal transporter (DMT1). Ferric iron reduction could also be affected by other metalloreductases (Steap proteins) and by non-enzymatic dietary reducing agents such as ascorbic acids [5].Unlike DMT1[6] and ferroportin [7], Dcytb lacks iron-response elements (IREs) even though it has been shown to be regulated by HIF2α and, indirectly, by hepcidin[8;9]. Shah *et al.* demonstrated the functional binding of HIF2α to hypoxia response elements (HREs) upstream of Dcytb (*sic.* CYBRD1) promoter region[8]. Moreover, Dcytb and DMT1 expression was down-regulated by hepcidin during inflammation[9].

Iron loading penetrance of hereditary haemochromatosis (HH) was reported to be high as levels of serum ferritin levels, which serve as markers of iron stores, were elevated in 82% of males and 55% of females []. HH disease penetrance, however, was only about 28% (in males) and 1% (in females) for human subjects that are homozygous for the C282Y mutation in the *HFE* gene[10]. Environmental and genetic factors [11] are proposed as modifiers of the expression of disease symptoms in C282Y homozygous patients. Phenotypic concordance of

iron overload symptoms has been reported in C282Y homozygote siblings[12;13]. Moreover, data from both animal and human studies have revealed associations between genetic variants of some genes of iron metabolism and symptomatic indices of iron loading in *HFE* homozygotes[14]. Considerable genetic variation exists in the severity of iron loading in HFE hemochromatosis, and Dcytb polymorphisms which possibly explains the different symptomatic phenotypes. Notably, a significant association was found between a single nucleotide polymorphism (SNP) rs3806562, located in the 5'UTR of CYBRD1, and transferrin saturation[15]. Quite significantly, the association between SNP rs884409, located in the promoter region of Dcytb, was associated with reduced serum ferritin[16]. Dcytb SNP rs10455 was also found in patients with iron overload who were also homozygous for a C282Y mutation in the HFE gene[16]. Dcytb rs10455, however, correlated positively with colorectal cancer[17]. As other potentially carcinogenic factors could result in iron overload, the precise and specific role of Dcytb rs10455 SNP was not evident in the phenotype of the disease in the patients studied[16]. In light of the above, it is imperative to study the functional activity of the rs10455 mutation in order to understand how it modifies iron overload phenotypes in human populations. The study therefore investigated ferric reductase activity of Dcytb SNP rs10455 mutation compared to that of the wild type in cultured transfected CHO cells.

Materials and Methods

Chemicals and reagents were obtained from Sigma-Aldrich or Merck (Dorset, UK) or from sources otherwise stated accordingly.

Cells and culture conditions

Chinese hamster ovary (CHO) cells were obtained from the American Type Culture Collection. Cells were cultured in Ham's F12 Nutrient Mixture medium (Sigma-Aldrich)

supplemented with 10% foetal calf serum, 4 mmol/l L-glutamine, 100 kU/L of penicillin, and 100 mg/L streptomycin. Cells were maintained at 37°C in a humidified incubator containing 95% air/5% CO_2. Cells were trypsinised, plated in 12- or 24-well plates, and grown for 24 h to full confluency for the experiments.

Transfection of cells

Human Dcytb gene fragment of 873 base pairs was synthesized (Genscript, USA) and cloned into a pcDNA (+) 3.1 myc/his(1) mammalian expression vector by BamHI and HindIII. The sequence of the Dcytb insert and the flanking sequences of the cloning sites were verified. Moreover, restriction digestion revealed the correct size of the insert as the only band on agarose gel electrophoresis. Cells were grown to 60–70% confluency andsubsequently transiently transfected with plasmid DNA using Fugene (Promega, USA) according to the manufacturer's protocol. Control cells were transfected with empty pcDNA 3.1 vector. Dcytb-expressing cells were selected with G418 and mixed colonies were used for the experiments.

Ferric reductase activity in CHO cells

CHO cells were grown to confluency in 12-well plates for ferric reductase assay. The assay buffer was prepared with 25 mmol/L 3-(*N*-morpholino)propanesulfonic acid (MOPS), 25 mmol/L 2-(*N*-morpholino)ethanesulfonic acid (MES), 5.4 mmol/l KCl, 5 mmol/L glucose, 140 mmol/L NaCl, 1.8 mmol/L $CaCl_2$ and 800 µmol/L $MgCl_2$. Additionally, 50 µmol/L $FeCl_3$ and 100 µmol/L nitrilotriacetic acid (NTA) and 200 µmol/L ferrozine were mixed and added to the MOPS assay buffer before incubation for 1 h at 37°C in the dark. Samples of assay buffer aliquots (200 µl) were read at 562 nm in the BioTek Synergy HT microplate reader. A ferrozine-based iron determination assay was performed (Reference needed here??). Standard curves were generated to convert the absorbance value into pmoles of iron reduced.

Endogenous reductase activity was measured using the protocol described above. Furthermore, cells were treated one day prior to the experiment with either 500 µmol/L (2,2,6,6-Tetramethylpiperidin-1-yl)oxyl (TEMPO), 100 µmol/L deferoxamine (DFO), 100 µmol/L $CoCl_2$, 100 µmol/L dehydroascorbic acid (DHA) or 100 µmol/L ferric ammonium citrate (FAC) following which ferric reductase assay was conducted.

Western blot analysis

CHO cells expressing WT, SNP Dcytb or empty plasmid were homogenized (in a buffer containing 0.1 mmol/L EDTA, 20 mmol/l KH_2PO_4, 135 nmol/L KCl and 1:200 (v/v) protease inhibitors at pH 7.4) with an Ultra Turrax (IKA, Staufen, Germany) homogenizer in (3 × 30 s pulses at full speed). The homogenate was centrifuged at 2000 rpm for 5 min. Following this,the supernatant was centrifuged for 1 h at 15,000 ***g*** to obtain the crude membrane fraction. Protein concentration was determined using Bio-Rad reagents (Bio-Rad, Laboratories, Hercules, CA, USA). Fifty (50) µg of membrane extracts were loaded onto a 12% gel in a SDS-PAGE. The proteins separated were then transferred to nitrocellulose membrane using a Bio-Rad dry transfer apparatus (Trans-BlotR SD Dry Transfer Cell; Bio-Rad, UK). Membranes were blocked with 5% milk for 1 h and probed with Dcytb Myc mouse antibody[7] (Santa Cruz Biotechnology, USA), and, β-actin (Sigma, UK) antibodies diluted in TBS. Cross-reactivity was observed with peroxidase-linked anti-IgG by using SuperSignal West Pico (Thermo Scientific, USA).

Statistical analysis

Statistical differences between means were calculated using Student's t test in correcting for differences in sample variance. When multiple comparisons were necessary, 1-way or 2-

way ANOVA was performed, using GraphPad Prism with Tukey's post hoc test. Differences were considered significant at $p< 0.05$.

Results

Dcytb SNP rs10455

The Dcytb rs10455 SNP investigated in the current study is a Ser266Asp missense mutation located in the last exon of Dcytb WT gene and the amino acid serine is highly conserved from zebrafish to human (Fig 1a). Western Blot analysis of Dcytb WT and SNP rs10455 protein expression in CHO cells is shown in Fig 1b.

Dcytb SNP rs14055 increases ferric reductase activity in CHO cells

CHO cells expressing Dcytb SNP rs10455 mutation exhibited significantly increased ferric reductase activity (Fig 2) compared to cells expressing Dcytb WT protein ($p<0.01$). Furthermore, both the SNP rs10455- and WT Dcytb-transfected CHO cells had significantly higher ($p<0.001$) ferric reductase activity than the control cells harboring the empty pcDNA plasmid only.

Deferrioxamine, FAC and $CoCl_2$ modulated ferric reductase activity in CHO cells

Following this, transgenic CHO cells expressing both the SNP rs10455 and WT Dcytb were pretreated overnight with DFO, FAC, or $CoCl_2$ (100 μmol/L) to simulate iron deficiency, iron loading or hypoxia. Ferric reductase activity was high in SNP rs10455 and WT Dcytb CHO cells than in cells with the empty plasmids (Fig 3). However, exposing cells to FAC overnight led to significantly reduced ferric reductase activity in all the three categories of samples (Fig 2). Consistently, CHO cells transfected with empty plasmid exhibited reduced ferric reductase activity when similarly exposed to the modulators of Dcytb expression.

Endogenous ferric reductase in CHO cells

Endogenous reductase activities of untransfected CHO cells were also investigated after cells were treated with potent modulators of Dcytb expression. To this end, CHO cells were treated with FAC, DHA, TEMPO, DFO or $CoCl_2$ and ferric reductase activity assays were compared against untreated cells (Fig. 4).

Iron chelation by DFO resulted in a significant ($p<0.05$) increase of ferric reductase activity (Fig 4). Treatment of CHO cells with $CoCl_2$, also enhanced ferric reductase activity, albeit to levels that were not significant statistically. However, iron loading of CHO cells with FAC reduced ferric reductase activity ($p<0.001$). Surprisingly, reductase activity was reduced in CHO cells exposed to either DHA ($p<0.01$) or the TEMPO radical ($p<0.05$) (Fig 4).

Discussion

Dcytb SNP rs14055 increases reductase activity

Duodenal cytochrome b (Dcytb) reduces ferric to ferrous iron for transport by DMT1 from apical membrane of the enterocytes. Dcytb SNP rs10455 was identified in a sample population of human HFE subjects and the functional analysis of the SNP was investigated in cultured CHO cells in the current study. The SNP is a genomic A<G mutation in the last exon of Dcytb gene which is a Ser266Asn amino acid substitution. The amino acid locus in the WT protein is highly conserved from zebrafish to humans (Fig 1a), thus suggesting a functional essentiality. Hence, the evolutionary conservation of rs10455 polymorphism indicates functional significance of the disease phenotype. The expression of the protein of both the WT and SNP variant in CHO cells was confirmed by Western blot analysis (Fig 1b). To investigate the function of the SNP rs10455, ferric reductase was performed in transgenic CHO cells harboring the WT or mutant Dyctb gene. Empty plasmid was transfected into CHO

cells and served as the control. The data revealed a gain of function of ferric reductase activity of CHO cells expressing Dcytb SNP rs10455 protein (Fig 2) above the WT and control, this implying a surfeit of ferrous ion substrate for transport by DMT1 and possibly in HFE subjects, an accentuation of iron-loading indices. The hematological status of HFE subjects with the Dcytb SNP rs10455 was, however not presented in the study by Constantine[11]. Strikingly, Davies *et al*. reported a positive correlation between SNP rs10455 and colorectal cancer in a genome-wide gene association study[17]. The missense polymorphism in Dcytb was associated with colorectal cancer in UK populations of UK English, but not those of Scottish, ancestry[17]. Reaction cascades and substrate channeling of ferrous iron availability, ROS generation and the induction of carcinogenesis are well documented[18]. Body iron stores and dietary iron intake have both been positively correlated with risk of colon cancer in some studies[19-21]. Relevant to the current study, plasma iron biomarkers and gender, rather than HFE gene mutations, were found to increase the risk of colorectal cancer and the development of polyps in a cohort of patients[22]. Consequently, multivariate risk factors could potentially contribute to colorectal cancer and Dcytb SNP rs10455 is a candidate polymorphism that could be screened in colon cancer patients.

In common with the findings in the current study, the loss-of-function phenotype of the Dcytb SNP rs884409 mutation was also associated[11] with HFE subjects. However, this SNP is located in the promoter region of Dcytb, and HFE subjects with this polymorphism had lower serum ferritin than was found in the control group. Moreover, functional luciferase reporter assay of Dcytb SNP rs884409 revealed a reduced basal reductase activity by about 30%. The consequent reduction of Dcytb expression concomitantly lowered iron absorption, to confer protection against iron loading that typifies HFE patients. Two other Dcytb SNPs found in

exon 1[23] rs 17554 and rs 3806562 transferrin saturation [14] were also reported to reduce the promoter activity in the gene[16]. These reports contribute to the reasons for varying (variable??) symptomatic or asymptomatic phenotypes in HFE genotype variants.

Dcytb is upregulated in hypoxia and iron deficiency

To further characterise the function of the Dcytb SNP rs10455, ferric reductase activity assays were conducted with modulators of Dcytb protein expression, notably the simulation of iron deplete, replete or hypoxia by FAC, DFO or $CoCl_2$ respectively (Fig 3). Ferric reductase activity was enhanced (Fig 3) in CHO cells that were exposed to DFO (iron chelation) or $CoCl_2$ (chemical hypoxia). Ferric iron reduction increased by either 60% or 40% in mutant CHO cells that were treated respectively with DFO or $CoCl_2$ (Fig 3). However, cells pretreated with iron loading exhibited a significant ($P<0.05$) 50% decrease in ferric reductase activity (Fig 3). The effects of FAC, DFO and $CoCl_2$ on ferric reductase activity were similar in the WT, SNP, untransfected categories as well as and in cells that were transfected with the empty plasmid (Figs 3 and 4).

Dcytb mRNA levels was shown to be down-regulated by treating cells with iron and dehydroascorbic acid[5]. Moreover, Dcytb expression was down-regulated in iron overload conditions[5;24] in the rat. The presence of dehydroascorbic in the assay medium, however, enhanced ferric reductase activity[5]. Dcytb is regulated by iron and hypoxia via the HIF2α[5;25] pathway. In normoxic conditions, iron- and oxygen-dependent HIF prolyl hydroxylases (PHD) mediate proteasomal degradation of HIF2α. However, during iron deficiency and hypoxia, PHD are inhibited so that HIF2α becomes stabilized and Dcytb expression is consequently enhanced[8].The regulation of Dcytb by HIF2α was demonstrated in the enterocytes of mice[8;25]. Furthermore, expression of both Dcytb and DMT1 was shown to be enhanced by

HIF2α during the early stages of exposure to hypoxia[26]. As observed previously[5;27], basal intrinsic ferric reductase activity could be high, variable in different cell types and, as seen in the current study, responsive to a number of modulating agents (Figs 3). Degeneracy and redundancy of ferric reduction ensue through both enzymatic and non-enzymatic processes in organs and tissues. The seemingly low expression of endogenous Dcytb protein (Fig. 1b), in contrast to high basal reductase activity (Figs 2-4), attests to the potential activities of other ferric ion 'reductases' in the cells. Dcytb could, therefore, be redundant from reducing agents such as ascorbate, glutathione, cysteine or superoxide radical, and possibly by SDR2 and Steap reductases as well. Significantly, however, Dcytb functionality is required particularly during conditions of enhanced iron absorption such as in hypoxia or cases of increased erythropoiesis[5;28].

In conclusion, this study provides evidence for a functional allelic association between Dcytb SNP rs10455 and ferric reductase activity in CHO cells. This gain-of-function polymorphism, might increase iron absorption in the gastrointestinal tract Future studies should now aim to elucidate underlying mechanisms of the SNP with relevance to the iron-loading phenotype of HFE subjects.

Legends

Figure 1a: SNP rs14055 is a highly conserved serine residue (red). (Adapted from http://web.expasy.org/variant_pages/VAR_038067.html).

Figure 1b: Western blot of Dcytb WT and SNP rs10455 protein in CHO cells. CHO cell lysates were separated by electrophoresis and blotted onto a nitrocellulose membrane.

Lanes are Dcytb, 1, SNP rs10455 2 and Empty plasmid 3. Membrane was stripped and re-probed with beta-actin antibody.

Figure 2: Ferric reductase activity assay of Dcytb WT and SNP rs14055. The assay was conducted in CHO cells overexpressing Dcytb WT, SNP rs10455 or in cells transfected with the empty plasmid. Ferrous iron reduction was normalized with protein content of the cells. Data shown are means of n=4±SE. Significance levels were determined by the two sample t-test for unequal variances (*p<0.05; (WT versus SNP rs10455) ** p<0.01; *** p<0.001(Empty versus WT/SNP rs10455).

Figure 3: Reductase activity assay of Dcytb WT and SNP rs10455 pre-treated with DFO, FAC or $CoCl_2$. CHO cells were overexpressing Dcytb WT (A), SNP rs10455 (B) or were transfected with the empty plasmid (C). Data shown are means of n=4±SE. Significance levels are shown for comparisons to untreated CHO cells as determined by the two sample t-test Empty versus WT/SNP (*** p<0.001) for DFO, $CoCl_2$ and FAC

Figure 4: Endogenous reductase activity assay of CHO cells. Untransfected cells were pre-treated with 100 µmol/L FAC, 100 µmol/L DHA, 500 µmol/L TEMPO, 100 µmol/L DFO or 100 µmol/L $CoCl_2$ one day prior to the assay. Ferrous iron reduction was normalized with protein content of the cells. Data shown are means of n=4±SE. Significance levels are shown for comparisons to untreated CHO cells as determined by the two sample t-test for unequal variances.). Untreated versus WT (FAC, p<0.001, DHA, p<0.005, Tempo, p<0.005, DFO, p<0.005, and $CoCl_2$ p<0.005.

Figure 5: Schematic representation of the physiological mechanism of non-haem iron absorption. Vectorial transport from the apical membrane in the small intestine into systemic circulation. Dcytb: Duodenal cytochrome b; DMT1: Divalent metal transporter 1; HCP1: Heme carrier protein 1; HO-1: Heme oxygenase 1; Hp: Hephaestin; LIP: Labile iron pool (Drawn by Christine Fischer, 2017).

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Human 246 RGSMPAYSGNNMDKS---DSELNSEVAARKRNLALDEAGQRSTM 286

Mouse 247 EGAIAISSAHSMDAADPADAESSSEGAARKRTLGLADSGQR 287

Rat 246 EGAIAISSAHNMDAA---DAELSSEGAARKRTLGLVDTGQR 283

Xenopus laevis 245 EGS-TITDCSNTEKS---DVELNSE-AARKRILKLDEAGQR 280

Xenopus tropicalis 245 EGS-TITDCSNTEKS---DVELNSE-AARKRILKLDDAGQR 280

Zebrafish 245 VGT---------------DMTTTS----------------- 253
```

Figure 1a **SNP rs10455 affects a highly conserved serine residue (red).** (Adapted from http://web.expasy.org/variant_pages/VAR_038067.html

	Total length of Dcytb (aa)	Position of conserved S
Human	286	266
Mouse	290	270
Rat	286	266
Xenopus laevis	283	264
Xenopus tropicalis	283	264
Zebrafish	253	253

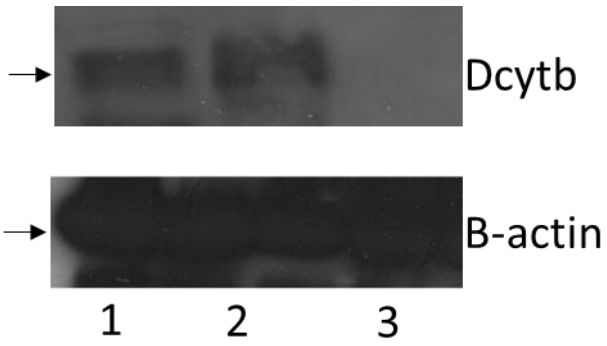

Figure 1b

1

2

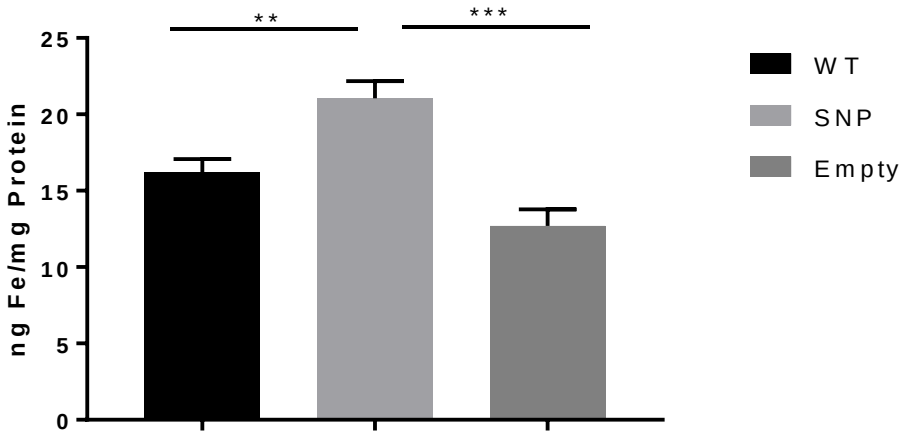

Figure 2

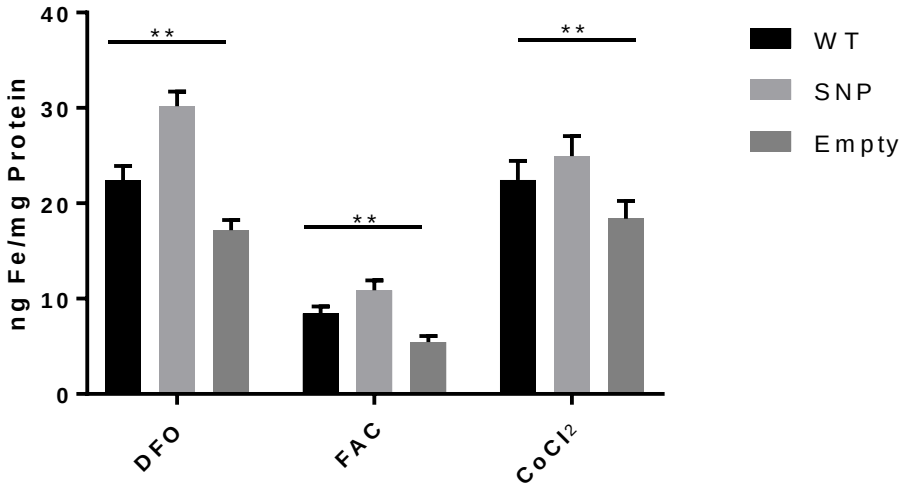

Figure 3

1

2

3

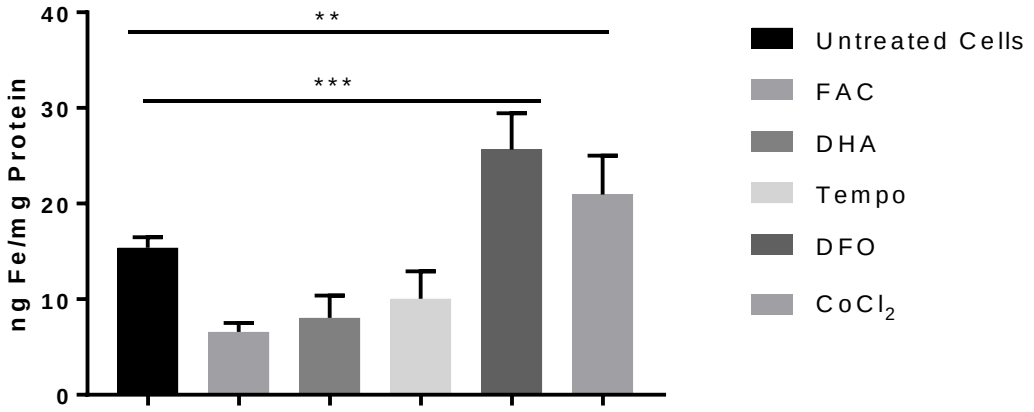

Figure 4

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